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13. ABSTRACT *(Maximum 200 words)*

We have developed and characterised a model of breast cancer metastasis to bone that overcomes the deficiencies of earlier models. It utilises clones of a spontaneous mammary carcinoma that, after orthotopic injection of the tumor cells into the mammary gland, metastasise to bone and cause hypercalcemia. In addition, metastases are detected in some other organs, mainly lungs and liver. Other clones derived from the same primary tumor either do not metastasise, or metastasise only to lungs. This is the only reported model in which the tumor cells spread spontaneously from the mammary gland to bone.

We have commenced studies, using this model to seek factors that are important in metastasis to bone. This is being done by two approaches: candidate gene analysis, whereby genes already implicated in bone and/or breast cancer metastasis are being examined; and genome wide screening for differences in gene expression between bone metastasising and non-bone metastasising clones. One candidate gene, parathyroid hormone related protein (PTHrP), is elevated both in the culture medium of the bone metastasising clone and in the serum of mice bearing these tumors. cDNA microarray screening has so far revealed a differential expression of several genes, including the α6 subunit of integrin.

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Robin L. Anderson 7/28/99

PI - Signature Date

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INTRODUCTION

Research into mechanisms of breast cancer metastasis to bone has been hampered by the lack of animal models that mimic the pattern of spread in humans. Most of the current models use immunocompromised mice and abnormal routes of introduction of the tumor cells to bone. In addition, until now, there has been no model that allows a comparison of primary tumors and secondary metastases in bone with the same primary tumor metastasising to another site. The aim of this project is to determine the factors that are important in the development of breast cancer metastases in bone. To achieve this aim, we have developed the first murine model of spontaneous metastasis of breast cancer cells to bone. We intend to fully characterise this model and use selected clones with defined metastatic patterns to seek genes that control metastasis to bone. This will be achieved by analysis of genes already implicated from other studies in the process and by genome wide screening for differentially expressed genes between clones that can or cannot metastasise to bone. When such factors are identified, it should be possible to develop specific therapies that target these factors and thereby reduce the incidence and morbidity associated with bone metastases.

BODY

The proposed specific aims of this project are as follows:

Task 1. To complete the characterization of the tumor model.

Task 2. To determine the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

Task 3. To identify the factors that are required for metastasis to bone:

- by analysis of candidate genes listed in Table 1 of the proposal.
- by genome wide screening for novel genes required for metastasis to bone.

Task 4. To modulate metastasis in vivo.

Task 5. To determine the mechanism of osteoclast mediated osteolysis by breast tumor cells.

It was agreed by the review panel that the proposal was too broad and that tasks 4 and 5 should be eliminated from the scope of the proposal. Hence, our efforts have focussed on the first three tasks.

Task 1: Characterisation of the tumor model

The exciting and innovative aspect of this proposal is our recent development of a novel murine model of metastatic breast carcinoma. The new model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer. In particular, these murine breast tumors metastasise to bone from the primary breast site

and cause hypercalcemia, characteristics not normally found in murine tumors, but common in the human disease. The model therefore provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone. The special advantage of this system is that it involves the whole metastasis process, beginning from the primary site. Existing models consider mechanisms that pertain to growth of tumor once the site has been reached (Price, 1996) (Kjønniksen et al., 1994) (Arguello et al., 1991).

The characterization of the model is largely complete and a manuscript is in press in Clinical and Experimental Metastasis. A summary of the findings reported in the manuscript is presented here and a copy of the proofs is included in the appendix.

The tumor cells used in this study are derived from a spontaneous mammary carcinoma in a BALB/c mouse (Aslakson and Miller, 1992). Originally, Dr. F. Miller provided us with three sublines of the spontaneous carcinoma that are not metastatic (67NR), that metastasise to the lungs (66cl4) or that metastasise to lungs and liver (4T1). The bone metastasising clone, 4T1.2, was derived by single cell cloning of 4T1 and was shown to metastasise to bone after orthotopic injection of 1 x 10^5 cells into the fourth mammary fat pad (See manuscript in the Appendix, Figure 1).

Growth and metastasis patterns of the clones.
The pattern of metastatic spread of 67NR, 66cl4 and 4T1.2 was assessed following inoculation into the mammary gland. Mammary tumors were palpable within 7-10 days. The tumors grew rapidly and the mice were killed when the tumors reached a weight of 1-2 g after 30-44 days. Some of the 4T1.2 tumor-bearing mice exhibited signs of partial paralysis of the hind limbs by this time. An analysis of primary tumor size, lung metastases and bone metastases is shown in Table 1 (see manuscript), with data from two independent experiments (Experiments 1 and 2). The tumor lines exhibited different growth rates *in vivo* that did not mirror their growth rates *in vitro*. 67NR, which has a slower growth rate *in vitro*, grew more rapidly *in vivo* than the other two lines. 4T1.2, which has a similar growth rate *in vitro* to 66cl4, grew more slowly *in vivo* than 66cl4. The 4T1.2 line showed a greater capacity to metastasise to the lungs from the mammary gland compared to 66cl4, whilst almost no tumor nodules were detected in the lungs of mice inoculated with 67NR (one mouse was found to have one lung tumor nodule in Experiment 1). In other experiments, all mice were culled on the same day after tumor inoculation and despite 4T1.2 primary tumors being smaller, the mice bearing 4T1.2 tumors still exhibited more lung nodules (data not shown). From visual examination of the mice, it is evident that 66cl4 tumors do not colonize other organs apart from the lung, whilst mice bearing 4T1.2 tumors often had visible nodules on the diaphragm and rib cage and enlarged lymph nodes. By histological examination of the spine and femur, only the 4T1.2 clone exhibited tumor growth in the bone. These observations indicate that, in addition to its ability to metastasise to bone, 4T1.2 has a greater capacity to metastasise to sites other than lung, compared to 66cl4.

Intracardiac injection of tumor cells.
To investigate further whether the 67NR and 66cl4 cells are unable to reach bone or whether they can reach, but not grow in the bone environment, tumor cells were injected into the left ventricle and hence into the arterial system leading to bone. Mice were assessed 11-17 days later when they first showed signs of distress (see manuscript, Table 1, Experiment 3). Whilst all three tumor lines showed an ability to colonise the lung, no histological evidence of tumor growth was found in the spine or femurs of four mice examined after inoculation of 66cl4 tumor cells. One of four mice examined had 67NR tumor growing in bone while two out of three mice carrying 4T1.2 tumor cells had tumor masses in the bones (manuscript Table 1). This indicates that the subline 66cl4 cannot proliferate in the bone environment whilst the non-metastatic 67NR clone, is capable of proliferating in this environment if forced there by arterial injection.

Histological analysis of primary and secondary tumors.
Primary tumors that were 10-15 mm in diameter showed areas of necrosis towards the central regions. All comprised sheets and cords of large undifferentiated cells typical of carcinoma (see manuscript, figure 2A). Large numbers of mitotic figures were also apparent. There were no obvious differences in morphology of tumors generated from the different cell lines. Pleural, subpleural and perivascular tumor nodules were evident in the lungs of mice bearing 66cl4 and 4T1.2 tumors. No tumor masses were detected in the livers of any animals although extramedullary hemopoiesis developed in livers of 4T1.2 tumor-bearing mice.

In the femora of 4T1.2 tumor-bearing mice, a single metastasis generally developed in the distal diaphysis, close to the growth plate and in some cases occupied large areas of the marrow space by 5 weeks post tumor inoculation (see manuscript, figure 2E, F). Tumors grew in the spines of 4T1.2 animals in multiple vertebrae and often involved surrounding muscle. Tumor growth in the spine led to complete replacement of bone marrow by tumor cells and invasion progressed into the spinal canal. Areas of active osteolysis could be seen adjacent to trabecular bone surfaces in both the spine and femur of 4T1.2 bearing animals (see manuscript, figure 2G, H).

Plasma calcium levels.
Circulating calcium levels were elevated in mice bearing 4T1.2 tumors, compared to those of mice bearing 67NR or 66cl4 tumors. Interestingly, there was a significant increase in plasma calcium in mice bearing 67NR tumors compared to non-tumor-bearing mice, but the value was lower than that in 4T1.2 tumor-bearing mice. No hypercalcaemia was evident in mice bearing 66cl4 tumors (see manuscript, Table 2).

Quantitation of tumor burden.
To assist in the quantitation of tumor burden in various organs, we have established stable cell lines expressing the reporter gene, green fluorescent protein (GFP). In the first approach, we used an expression vector in which GFP expression was driven by the CMV promoter, but we found that, *in vivo*, GFP expression became erratic, with expression turned off in some tumor cells. To correct this, we switched to the EF-1α

promoter, which is commonly used to drive exogenous genes in transgenic mice. Here, we found much stronger GFP expression, but slower tumor growth rates. We postulate that presentation of GFP peptides on Class 1 MHC has caused an immunological anti-tumor response. As a compromise, we are now using a poorly fluorescent 4T1.2/GFP clone that has the same growth characteristics as 4T1.2 and are developing a quantitative PCR assay to measure tumor burden in various organs. Preliminary data indicate that the technique appears to be working well.

Task 2. Determination of the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

The kinetics of metastatic spread to bone of three sublines, 67NR, 66cl4 and 4T1, and the clonal line 4T1.2 derived from the original spontaneous carcinoma, were assessed in female BALB/c mice of 6-8 weeks of age following varying routes of inoculation. Micrometastases were first detected in the lung on day 15 after inoculation of 4T1 cells into the mammary fat pad. By day 22, micrometastases were first detected in the vertebrae but only with 4T1 cells. Tumor cells were never recovered from the vertebrae of mice inoculated with 67NR or 66cl4. Thus our data indicate that the non-bone metastasising lines also fail to home to the bone environment in numbers detectable in our assay.

After intravenous injection, clonogenic 4T1 cells were recovered from lungs at all times. Lung metastases become grossly visible by day 19. Vertebrae became uniformly positive by day 17 following injection. An attempt to isolate a clone of 4T1 for which clonogenic cells were not detected in the vertebrae was unsuccessful. Twenty clones were randomly isolated and injected intravenously. Although some clones appeared to be quantitatively more metastatic to bone than others, clonogenic cells were recovered from spines by day 20 in all cases. Subsequent analyses of overt tumor growth were performed using clone 2 of 4T1 (4T1.2). Thus, the appearance of overt tumors in bone correlated with micrometastatic deposits and no micrometastases were recovered from clones unable to form overt tumors in bone.

Task 3. Identification of the factors that are required for metastasis to bone, both by analysis of candidate genes listed in Table 1 of the proposal and by genome wide screening for novel genes required for metastasis to bone.

(i) Analysis of candidate genes

We have proposed a list of candidate genes to investigate for their role in bone metastasis (see proposal, Table 1). There is evidence in the literature that implicates each of these genes in breast cancer metastasis and/or bone metastasis. We have commenced investigations on some of these, including parathyroid hormone-related protein (PTHrP), estrogen receptor (ER), urokinase plasminogen activator (uPA), matrix metalloproteinase-2 and -9 (MMP2 and MMP9). However, most data we have obtained so far relates to the role of PTHrP in bone metastasis.

PTHrP

Elevated PTHrP has been implicated in promoting hypercalcemia and bone metastasis in several xenograft models (Guise et al., 1996). Primary tumors generated by all three cell lines stained positively for PTHrP (see manuscript, figure 2C). In tumors from 66cl4 and 4T1.2 cells, staining was usually most intense in dividing cells with mitotic figures, and at the edges of the tumors where normal tissue invasion was occurring. Tumors from 67NR cells tended to stain more weakly, with mitotic cells weakly positive or negative for PTHrP. Metastases in lungs also stained weakly positive for PTHrP. In both the femur and spine, 4T1.2 tumors were positive for PTHrP but there was no apparent difference in intensity from that seen in the tumor at the primary site (see manuscript, figure 2E, F).

However, differences were observed when the amount of secreted PTHrP was measured. PTHrP levels were measured both in the medium of cultured cells and in the plasma of tumor-bearing mice. PTHrP secretion into the medium of the tumor sublines grown in culture was measured in exponentially growing cells placed in serum-free medium for 20h. As shown in Figure 3 of the manuscript, PTHrP levels were highest in the 4T1.2 line, intermediate in 66cl4 cells and negligible in 67NR cells. Plasma PTHrP levels in tumor-bearing mice are shown in Table 2 of the manuscript. Compared to the value in non-tumor-bearing mice, plasma PTHrP was elevated approximately 1.7 fold in mice bearing 4T1.2 tumors ($p<0.04$ when analysed using a Student t test). In parallel, plasma calcium levels were elevated in mice bearing 4T1.2 tumors (see manuscript, Table 2).

Current experiments are focussed on the regulation of PTHrP expression *in vitro* and on the consequences in terms of metastatic spread of altered PTHrP expression *in vivo*. By examination of 34 single cell clones of 4T1 (of which 4T1.2 is one such clone), we have found clones with differing PTHrP secretion levels. We are analysing the ability of these clones with disparate PTHrP secretion to metastasise to bone after mammary fat pad injection. In addition, using sense and anti-sense expression constructs for PTHrP, we have generated lines derived from two of these clones that have elevated or suppressed PTHrP secretion *in vitro*. We are currently evaluating their growth and metastatic capacity in mice as well. Preliminary results indicate that suppression of expression of PTHrP reduces the extent of bone metastasis in the 4T1 line. In contrast, over-expression of PTHrP in the non-bone metastasising 66cl4 did not enable these cells to proliferate in bone.

Estrogen receptor

By immunohistochemistry, we have shown that all the sublines express the estrogen receptor. Future experiments will examine the dependence of the sublines of estrogen for growth and metastatic capacity.

Plasminogen activator

In collaboration with Dr. E. Allan (St. Vincent's Institute of Medical Research), we have measured the activity of the plasminogen activators, uPA and tPA, in the sublines with differing metastatic capacity. uPA and tPA are serine proteases that are involved in cell migration and tissue remodelling. uPA is frequently expressed at high levels in tumors and can enhance bone metastases in prostate cancer (Rabbani et al., 1999). We have found that the bone metastasising clone, 4T1.2, expresses significantly higher levels of uPA and tPA than the other sublines. We are planning gain-of-function experiments similar to those underway for PTHrP; specifically by altering expression of uPA in the sublines and following their metastatic capacity *in vivo*.

Matrix metalloproteinases

In collaboration with Dr. E. Thompson (St. Vincent's Institute of Medical Research), we have been analysing the expression and activation of MMP2 and MMP9 and the *in vitro* invasive properties of the sublines. These enzymes degrade collagen, a major component of the extracellular matrix (Stetler-Stevenson et al., 1993). In Boyden chamber assays, invasion through Matrigel was more pronounced for 4T1.2 cells, with 66cl4 showing a moderate rate of invasion and 67NR cells, a low level. Migration over collagen (type 1 or IV) was low in both 67NR and 66cl4 compared to 4T1.2 cells. The 4T1.2 subline showed enhanced activity of some of the matrix metalloproteinases (MMP). Specifically, MMP9 secretion and MMP2 activation were both higher in 4T1.2 compared to 66cl4 or 67NR, which may allow 4T1.2 a greater capacity to degrade the extracellular matrix of bone.

(ii) Genome wide screening for genes differentially expressed in bone metastasising clones.

We have initiated a number of studies in this area, but as we are only in the first year of the project, definitive results are not yet available. Since writing the initial proposal in which we stated an intention to use differential display (DD) or representational difference analysis (RDA), a variety of cDNA array techniques have been developed. We decided to investigate two of these techniques as potentially they can yield considerable information in a short time span. We obtained access, through Dr. David Bowtell, to a cDNA microarray chip developed at the NIH. This chip has 1200 mouse genes and ESTs arrayed on it. In parallel, we purchased cDNA membrane filters from Genome Systems. These filters array approximately 18,000 mouse genes and ESTs on a filter. RNA was isolated from both cultured cells and primary tumors of the two sublines 4T1.2 and 66cl4 and screened for differential expression using the filters and gene chip. For the chip, the RNA was reversed transcribed into cDNA and tagged with either Cy5 (green) or Cy3 (red) fluorescent dyes. Equal amounts of tagged cDNA from the two samples were competitively hybridized to the chip. If equal amounts of a specific RNA were present in the two samples, the spot will fluoresce yellow (equal green and red dyes). Differential expression will appear as either green or red fluorescence over a particular spot. An example of the results from this approach is shown in Figure 4A.

The cDNA filter approach is different in that two matched filters are used, one for each sample. During reverse transcription of the RNA to cDNA, a ^{33}P-labelled nucleotide is incorporated. After hybridization of the cDNA to the filter, signals are measured by exposure of the filter to a phosphoimager screen. Signal intensities for each spot can be quantitated and the difference compared between the two filters bearing RNA derived from 4T1.2 or 66cl4 cells or tumors. An example of the results from the filters is shown in figure 4B.

The results from these two techniques can be summarized as follows:

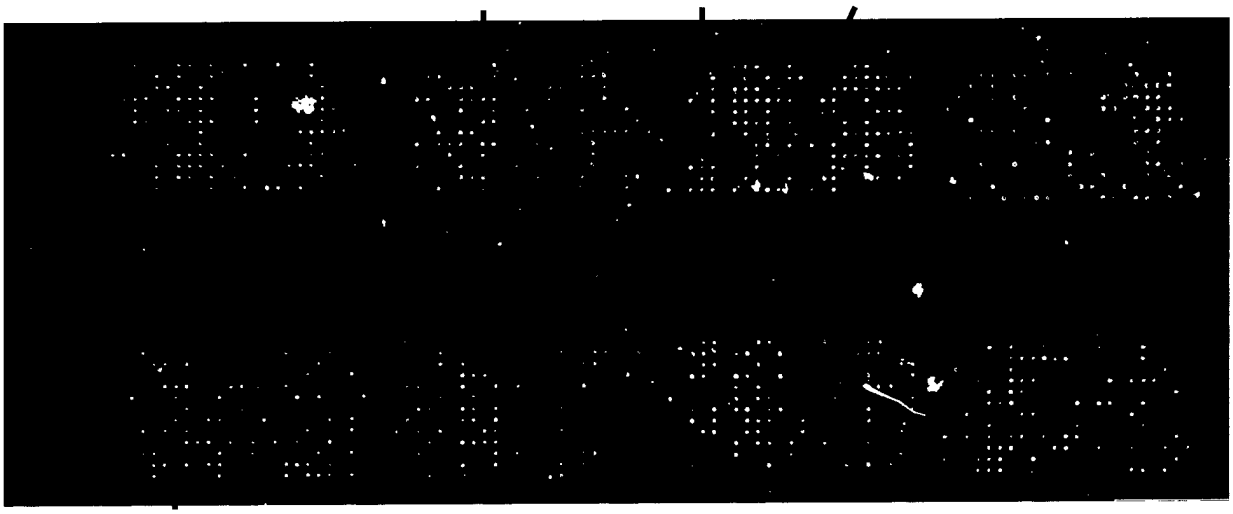
(i) It was noted that the two tumor sublines are indeed well matched genetically as only a limited number of differences in expression were noted.

(ii) Reproducibility of results using the filters was a problem with different signals showing up as being differentially expressed in repeat experiments.

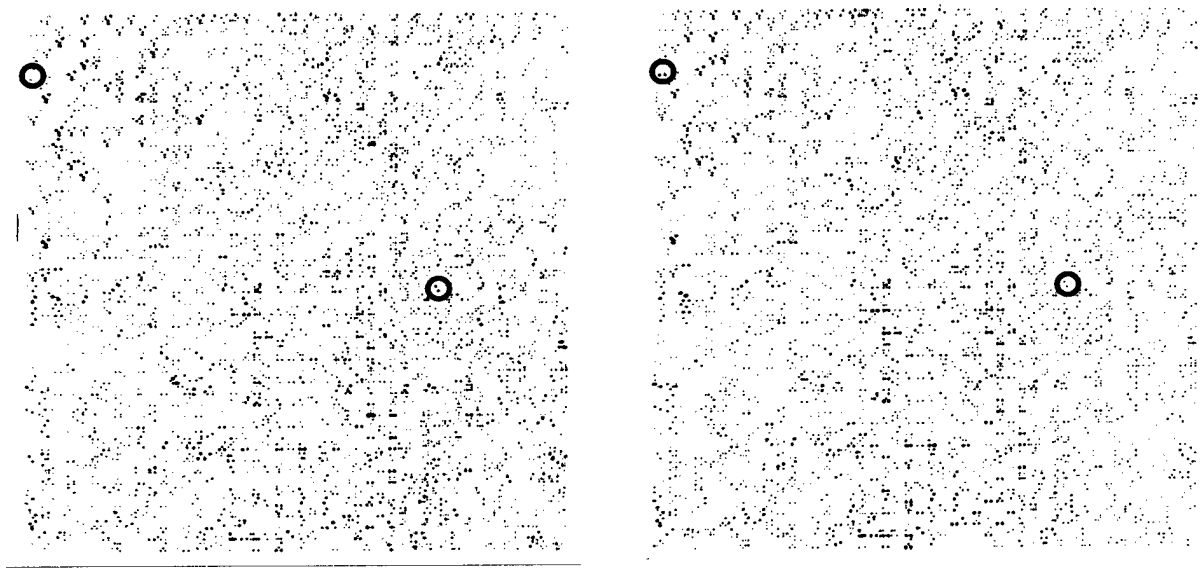
(iii) The identity of the clones, as supplied by the manufacturer, was not always correct.

(iv) From the two approaches, 30 clones that appeared to be differentially expressed were selected and purchased. Verification of identity was obtained by sequencing and of differential expression by northern analysis. The differential expression of only one (α6 integrin) of the 30 clones identified from the DNA microarray or the cDNA filter arrays has so far been confirmed by northern analysis.

In conclusion, these two techniques, whilst confirming α6 integrin as being differentially expressed, have not yielded sufficiently reproducible results for us to continue selecting differentially expressed genes for further analysis. We now intend to pursue the original proposal of using RDA and are investigating the possibility of linking RDA to subsequent DNA microarray analysis of differentially expressed genes. This should give us a rapid means of discriminating between false positives and genes that really are differentially expressed.



DNA Microarrays.
DNA microarrays containing 1200 known genes were probed with Cy5-labelled cDNA (green) from 4T1.2 and Cy3-labelled cDNA from 66cl4 (red). Genes more highly expressed in 4T1.2 are represented by green spots, those more highly expressed in 66cl4 by red spots and those equally expressed by yellow spots.



cDNA Arrays.
Genome Systems mouse Gene Discovery Arrays were probed with ^{33}P-labelled cDNA from 4T1.2 (left) and 66cl4 (right). Differentially expressed genes are indicated by coloured circle pairs.

KEY RESEARCH ACCOMPLISHMENTS

- Discovery of a murine breast carcinoma line that metastasises from the mammary gland to bone.
- Characterization of clones derived from the original carcinoma, which have differing metastatic potentials.
- Demonstration that the appearance of micrometastases in bone marrow correlates with the subsequent development of overt bone tumors.
- Demonstration that increased levels of PTHrP secretion, uPA activity, MMP9 activity and MMP2 activation correlate with the ability to metastasise to bone.

REPORTABLE OUTCOMES

- Manuscript in press in *Clinical and Experimental Metastasis.*
  Lelekakis, M., Moseley, J.M., Martin, T.J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F.R., Slavin, J., Anderson, R.L. (1999) A novel orthotopic model of breast cancer metastasis to bone. Clin. Exp. Metastasis (in press).

- Abstract and poster presentation to the 7th International Congress of the Metastasis Research Society, 1998.
  A Model of spontaneous breast cancer metastasis to bone. Anderson, R.L., Lelekakis, M., Miller, F., Williams, E.D., Hards, D., Martin, T.J. and Moseley, J.M.

- Abstract, poster and platform presentation to the Keystone Symposium entitled Molecular Pathogenesis of Bone Disease, California, 1999.
  Characterization of a novel murine model of breast cancer metastasis to bone. Tavaria, M., Sloan, E., Lelekakis, M., Ho, P., Hards, D., Williams, E., Martin, T.J., Moseley, J. and Anderson, R.L.

- Keystone travel grant awarded to M. Tavaria for the best short platform presentation.

- B. Med. Sc. degree awarded to Mr. Marcus Foo based on his thesis that investigated the relationship between the presence of metastatic cells in bone marrow and the development of overt bone tumours.

- The development and characterization of the only murine model of spontaneous metastasis from the mammary gland to bone. This model offers great potential in testing novel therapeutics targeted at bone metastases.

CONCLUSIONS

The major achievement over the past year has been the characterization of the murine breast cancer model of metastasis from the mammary gland to bone. To our knowledge, this is the only model that mimics the human disease and it provides a unique opportunity to search for the genes that control metastasis to bone. We have demonstrated that only in the bone metastasising clone do we find metastatic cells in the bone marrow, implying that the other clones cannot home to bone. By the use of intracardiac injections, we have shown that the non-metastatic clone can proliferate in bone when forced there by arterial blood flow, but the lung metastasising clone cannot proliferate in bone marrow.

We have begun to investigate several genes already implicated in breast cancer metastasis and/or bone metastases and have found elevated expression and/or secretion of PTHrP, MMP9, MMP2 and uPA. Future experiments will focus on manipulating the expression of these genes and testing the consequences in terms of metastatic capacity. From our limited genome screening for differentially expressed genes, we have found elevated expression of the integrin $\alpha 6$ in the bone metastasising clone.

The expression of any genes found here to be important in directing breast cancer cells to bone and/or in controlling their proliferation in bone will ultimately be measured in archival specimens of human breast cancer and metastases. If the altered expression patterns are also found in the human disease, therapeutic strategies will be devised to specifically target these genes, hopefully leading to reduced metastatic burden in the bones of breast cancer patients. The model also has great value in testing new therapeutic reagents developed by others, for their efficacy against metastatic breast cancer.

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APPENDIX:

A copy of the proofs of the article about to be published in *Clinical and Experimental Metastasis*.

Clinical & Experimental Metastasis **0:** 1–8, 1999.

A novel orthotopic model of breast cancer metastasis to bone

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Abstract

Breast cancer affects approximately one woman in twelve and kills more women than any other cancer. If detected early, patients have a five year survival rate of 66%, but once metastatic disease has developed, there is no effective treatment. About 70% of patients with metastatic disease have bone involvement, while lungs and liver are the other common targets. Bone metastases cause severe pain, pathological fractures and hypercalcaemia and thus are a significant clinical problem. The development of new therapies for metastatic breast carcinoma depends on a better understanding of the mechanism of homing of the tumour cells to bone, liver and lungs and the factors required for their growth in these organs.

Research on mechanisms of breast cancer metastasis, particularly to bone, has relied on *in vitro* studies or on tumour models in which the inoculation route is designed to promote delivery of tumour cells to a specific organ. Metastases in bone are achieved by inoculation into the right ventricle of the heart. To our knowledge there has been no report of a model of metastatic spread from the mammary gland to distant sites which reliably includes bone. In this paper, we describe our recent development of a novel murine model of metastatic breast carcinoma. The new model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer. In particular, these murine breast tumours metastasise to bone from the primary breast site and cause hypercalcaemia, characteristics not normally found in murine tumours, but common in human disease. Furthermore, in a preliminary characterisation of this model, we show that secretion of parathyroid hormone-related protein, a role for which has been implicated in breast cancer spread to bone, correlates with metastasis to bone.

This model therefore provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone. The special advantage of this system is that it involves the whole metastasis process, beginning from the primary site. Existing models consider mechanisms that pertain to growth of tumour once the site has been reached. An understanding of the regulation of these factors by potential therapeutic agents could lead to improvement in therapies designed to combat metastatic disease. For the first time, this development will allow exploration of the molecular basis of site-specific metastasis of breast cancer to bone in a clinically relevant model.

Introduction

Bone metastases are a major cause of morbidity in breast cancer and occur in 70% of patients who develop metastatic disease. Hypercalcaemia is a common complication due to excessive bone resorption which is caused by increased numbers of active osteoclasts. This leads to the breast cancer patient suffering severe pain and bone fractures [1]. The preference of breast tumour cells for growth in bone is not fully understood, but is undoubtedly facilitated by their ability to stimulate adjacent osteoclasts to resorb bone. This process is promoted by the ability of these tumour cells to express bone compatible matrix proteins and adhesion molecules and by tumour cell interactions with local growth factors in bone. Several factors that have normal roles in bone turnover and metabolism have been detected in primary breast cancers. These include parathyroid hormone-related protein (PTHrP), tumour necrosis factor-α (TNFα), transforming growth factor-β (TGFβ) and prostaglandins [1] [2], interleukin 6 (IL6) [3], interleukin 11 (IL11) [4], osteopontin [5] [6] and bone sialoprotein [6]. The ability of these factors to alter bone turnover and to be regulated in the bone environment may facilitate establishment of tumours in bone.

The development of animal models of human tumour cells injected into immunodeficient mice for the study of breast cancer spread to bone has provided strong evidence for the importance of PTHrP in the establishment of bone

metastases [7]. However, these models are dependent upon the injection of tumour cells into the left ventricle of the heart in order to promote tumour cell delivery to bone and therefore can only evaluate factors important for invasion of bone and interaction of tumour cells with the bone environment. Until now, no models of spontaneous metastasis of breast tumour cells from the primary site to bone have been reported, although orthotopic inoculation has been shown to result in development of secondary tumours in lymph nodes and lungs [8]. Breast cancer cells can be forced to the site of interest, for example by intravenous injection to promote growth in lungs, into the portal vein to produce liver tumours, and into the carotid artery to induce brain tumours [8]. To induce tumour cell growth in bone, cells can be injected directly into the tibia [9] or into the arterial system [10]. However, all of these routes result in a bolus of cells arriving in bone or other organ of interest, rather than the situation that occurs in clinical disease, in which cells escape gradually over time from the primary tumour and circulate through the vascular system or lymphatics before colonizing a distant organ.

We report here the development of a novel model of metastasis from the mammary gland to bone using a clonal tumour line derived from a spontaneously arising mammary tumour in a BALB/cfC3H mouse [11]. The isolation of sublines that are either non-metastatic, or that metastasise to lungs or that metastasise to lungs and liver has been described previously [12]. Here we report that one of the subpopulations, 4T1, also metastasises to bone. We have further characterised a single cell clone derived from 4T1 (4T1.2) that metastasises to bone and lung. The model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer, causing osteolytic lesions in bone. Given the documented role of PTHrP in bone lesions of human breast cancer, we have examined the expression patterns of PTHrP both *in vitro* and *in vivo* and have found that both primary tumours and bone lesions of the bone metastasising clone express this growth factor.

Materials and methods

Cell culture

Four sublines of breast cancer cells, 67NR, 66cl4, 4T1 and 4T1.2, derived from a spontaneous carcinoma in a Balb/cfC3H mouse were used. The derivation of the first three of these sublines has been described previously [12]. Briefly, 67NR is a geneticin resistant variant derived from subpopulation 67, 66cl4 is a thioguanine/ouabain resistant variant of subpopulation 66 and 4T1 is a thioguanine resistant variant of subpopulation 410.4. Clone 4T1.2 was derived by single cell cloning of 4T1 (Figure 1).

The tumour lines were maintained in a 5% CO_2 incubator in MEM (alpha modification) containing 10% FCS and antibiotics (penicillin and streptomycin). They were checked regularly for mycoplasma contamination using the Gene-Probe kit. To minimise genetic drift, the cells were maintained in culture for no more than four to six weeks.

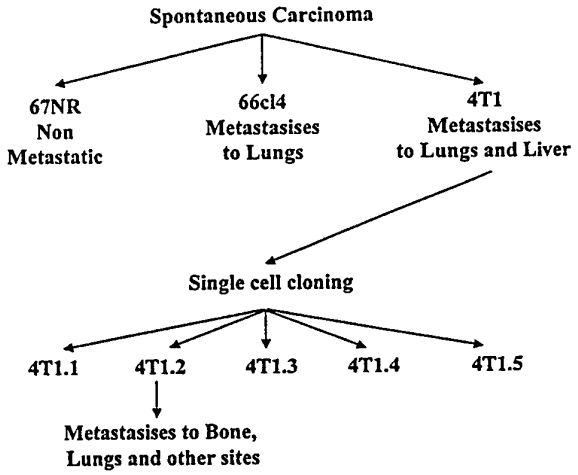

Figure 1. Flow chart showing the derivation of the breast tumour lines used in this study.

Prior to inoculation, cells were checked for viability using trypan blue.

In vivo *studies*

Female BALB/c mice aged 6–8 weeks were injected into the fourth mammary fat pad with 1×10^5 viable cells in a 10 μl volume, or into the lateral tail vein with 5×10^5 cells in a 0.2 ml volume, or into the left ventricle with 2×10^5 cells in a 0.2 ml volume.

Spontaneous metastasis from the mammary fat pad

Tumours were palpable 7–10 days after injection into the mammary fat pad. Primary tumour size was measured three times a week using callipers and the final tumour weight was measured after sacrificing the mice and excising tumours. Tumour burden in the lungs was quantitated by counting nodules after inflation of the lungs with India ink. Metastases in other organs were observed visually or by histological examination of formalin fixed samples. Blood was collected by cardiac puncture under anaesthetic at the time of sacrifice to obtain plasma for PTHrP and calcium assays. In some experiments, occult metastatic cells were quantitated by recovery of clonogenic cells from disaggregated tissues and growth in selective media as described previously [12]. By sacrificing groups of 5–6 animals at different times after initiation of the primary tumour, the kinetics of metastasis to the lung and bone were determined. Lungs were digested as previously described [12]. The spinal column was removed, individual vertebrae split open with scalpels, agitated, and stirred briefly in media containing 60 μM 6-thioguanine. The crushed spinal column was removed and the released cells were incubated for 7–10 days at 37 °C before colonies were fixed, stained, and counted.

Kinetics of bone colonisation following intravenous injections

Groups of 3–6 mice were sacrificed 7, 12, 17 and 19 days after intravenous injection of 5×10^5 tumour cells. Lungs were

enzymatically digested and vertebral columns were crushed and released cells plated in selective media as described above.

Bone metastasis following intracardiac injections

Tumour cells were injected into the left ventricle and hence directly into the arterial system. Mice were culled when they first showed signs of distress. The number of lung metastases was assessed by India ink inflation and the presence of tumour deposits in the bone was detected by examination of histological sections of the spine and femur.

Histology

Primary tumours, lungs, liver, femora and spines from tumour-bearing and control mice were fixed in 10% buffered formalin. Bones were decalcified in EDTA [13] and embedded in paraffin. Standard haematoxylin and eosin (H&E) staining procedures were employed for assessment of morphology. For detection of bone metastases, the paraffin embedded samples were cut into 5 μm sections and every twentieth section was stained and examined by microscopy.

Immunohistochemistry

PTHrP. PTHrP immunostaining was carried out as previously described [14] using a sheep polyclonal antibody raised against synthetic human PTHrP (50–69) at dilutions of 1/200 and 1/300 overnight at 4 °C. Normal sheep serum was used for non-immunecontrols and normal mouse skin was used as a positive control in all assays. Other controls included deletion of each of the reagents.

Keratin. A rabbit polyclonal anti-keratin (wide spectrum 2622 from Dako Corp.) was used overnight at dilutions of 1/500 and 1/750 at 4 °C. Antigen retrieval was achieved by microwave treatment in 0.05 M tris buffer pH10 and detection was by biotin-streptavidin-peroxidase.

Estrogen receptor. A monoclonal antibody against the human estrogen receptor (ER) (Novocastra NCL-ER-6F11), which cross-reacts with the mouse protein, was used overnight at dilutions of 1/250 and 1/500 at 4 °C. Antigen retrieval was achieved by microwave exposure in 0.01M citrate buffer, pH6 and detected with biotin-streptavidin-peroxidase.

In all cases, specific reaction is indicated by brown staining in the sections.

Radioimmunoassay for PTHrP

PTHrP was measured with an N-terminal directed antibody using a radioimmunoassay described previously [15]. To assay release of PTHrP from cultures, cells in an exponential phase of growth were incubated in serum-free medium containing 0.1% BSA for 1 h. The medium was then replaced with fresh serum-free medium containing 0.1% BSA for 20 h. PTHrP was measured on aliquots of the medium cleared of cell debris by centrifugation. To correct for cell numbers, the remaining monolayer of cells was rinsed with PBS, drained, dissolved in 1M sodium hydroxide overnight at 37 °C and the protein content measured using a modified Lowry assay.

Plasma calcium assay

Total calcium was measured using the Arsenazo III assay (Trace Scientific, Australia), following the recommended protocol.

Results

The kinetics and patterns of metastatic spread of three sublines, 67NR, 66cl4 and 4T1, derived from the original spontaneous carcinoma (Figure 1), were assessed in female BALB/c mice of 6–8 weeks of age following varying routes of inoculation. In addition, we have characterised a single cell clone of 4T1, called 4T1.2, that shows a strong propensity to metastasise to bone. In a previous analysis, 67NR was characterised as being non-metastatic, 66cl4 as metastasising to lung and 4T1 as metastasising to lung and liver [12].

Kinetics of 4T1 metastasis

After inoculation into mammary gland, clonogenic 4T1 cells were always recovered from the lungs prior to recovery from the bone. Clonogenic cells were recovered by day 15 (first day assessed) from the lungs but not from bone at a time when the median weight of the primary tumours was 216 mg. Clonogenic 4T1 cells were not recovered from bone at day 19 (6 mice tested) when the median weight of the primary tumours was 343 mg, but were recovered from vertebrae of 5 of 6 mice at day 22 when the median weight of the primary tumours was 546 mg. In similar experiments, clonogenic cells were not recovered from vertebrae of mice bearing 67NR or 66cl4 primary tumours. After intravenous injection, clonogenic 4T1 cells were recovered from lungs at all times. Lung metastases become grossly visible by day 19. Vertebrae were uniformly negative at days 7 and 12, but became uniformly positive by day 17 following injection. An attempt to isolate a clone of 4T1 for which clonogenic cells were not detected in the vertebrae was unsuccessful. Twenty clones were randomly isolated and injected intravenously. Although some clones appeared to be quantitatively more metastatic to bone than others, clonogenic cells were recovered from spines by day 20 in all cases. Subsequent analyses of overt tumour growth were performed using clone 2 of 4T1 (4T1.2).

Analysis of growth patterns of the breast carcinoma sublines

The pattern of metastatic spread of 67NR, 66cl4 and 4T1.2 was assessed following inoculation into the mammary gland. Mammary tumours were palpable within 7–10 days. The

tumours grew rapidly and the mice were killed when the tumours reached a weight of 1–2 g after 30–44 days. Some of the 4T1.2 tumour-bearing mice exhibited signs of partial paralysis of the hind limbs by this time. An analysis of primary tumour size, lung metastases and bone metastases is shown in Table 1, with data from two independent experiments (Experiments 1 and 2). The tumour lines exhibited different growth rates *in vivo* that did not mirror their growth rates *in vitro*. 67NR, which has a slower growth rate *in vitro*, grew more rapidly *in vivo* than the other two lines. 4T1.2, which has a similar growth rate *in vitro* to 66cl4, grew more slowly *in vivo* than 66cl4. The 4T1.2 line showed a greater capacity to metastasise to the lungs from the mammary gland compared to 66cl4, whilst almost no tumour nodules were detected in the lungs of mice inoculated with 67NR (one mouse was found to have one lung tumour nodule in Experiment 1). In other experiments, all mice were culled on the same day after tumour inoculation and despite 4T1.2 primary tumours being smaller, the mice bearing 4T1.2 tumours still exhibited more lung nodules (data not shown). From visual examination of the mice, it is evident that 66cl4 tumours do not colonize other organs apart from the lung, whilst mice bearing 4T1.2 tumours often had visible nodules on the diaphragm and rib cage and enlarged lymph nodes. By histological examination of the spine and femur, only the 4T1.2 clone exhibited tumour growth in the bone, confirming the assays for clonogenic tumour cells mentioned above.

Intracardiac injection of tumour cells

To investigate further whether the 67NR and 66cl4 cells are unable to reach bone or whether they can reach, but not grow in the bone environment, tumour cells were injected into the left ventricle and hence into the arterial system leading to bone. Mice were assessed 11–17 days later when they first showed signs of distress (Table 1, Experiment 3). Whilst all three tumour lines showed an ability to colonise the lung, no histological evidence of tumour growth was found in the spine or femurs of four mice examined after inoculation of 66cl4 tumour cells. One of four mice examined had 67NR tumour growing in bone while two out of three mice carrying 4T1.2 tumour cells had tumour masses in the bones (Table 1).

Histological analysis of primary and secondary tumours

Primary tumours that were 10–15 mm in diameter showed areas of necrosis towards the central regions. All comprised sheets and cords of large undifferentiated cells typical of carcinoma. Large numbers of mitotic figures were apparent. There were no obvious differences in morphology of tumours generated from the different cell lines (see Figure 2A for the 4T1.2 primary tumour). Pleural, subpleural and perivascular tumour nodules were evident in the lungs of mice bearing 66cl4 and 4T1.2 tumours (not shown). No tumour masses were detected in the livers of any animals although extramedullary hemopoiesis developed in livers of 4T1.2 tumour-bearing mice (not shown).

In the femora of 4T1.2 tumour-bearing mice, a single metastasis generally developed in the distal diaphysis, close to the growth plate and in some cases occupied large areas of the marrow space by 5 weeks post tumour inoculation (Figures 2E, F). Occasionally, attached muscle adjacent to the bone metastasis became involved. Tumours grew in the spines of 4T1.2 animals in multiple vertebrae and often involved surrounding muscle (Figure 2G). Tumour growth in the spine led to complete replacement of bone marrow by tumour cells and invasion progressed into the spinal canal. Areas of active osteolysis could be seen adjacent to trabecular bone surfaces in both the spine (Figure 2H) and femur (not shown) of 4T1.2 bearing animals.

PTHrP, keratin and ER expression in primary and secondary tumours

Primary tumours generated by all three cell lines stained positively for PTHrP (see Fig.2C for 4T1.2). In tumours from 66cl4 and 4T1.2 cells, staining was usually most intense in dividing cells with mitotic figures, and at the edges of the tumours where normal tissue invasion was occurring. Tumours from 67NR cells tended to stain more weakly, with mitotic cells weakly positive or negative for PTHrP (not shown). Metastases in lungs also stained weakly positive for PTHrP. In both the femur and spine, 4T1.2 tumours were positive for PTHrP but there was no apparent difference in intensity from that seen in the tumour at the primary site (Figures 2C and E). For comparison, the PTHrP staining of a 4T1.2 tumour in femur and a non-immune control are shown (Figures 2E and F, respectively). Strong staining for keratin and weaker staining for the ER was evident for all three primary tumour types (see Figure 2B and D for keratin and ER immunostaining in 4T1.2 tumours, respectively). 4T1.2 tumours in bone also stained positively for keratin (not shown).

PTHrP secretion

PTHrP levels were measured both in the medium of cultured cells and in the plasma of tumour-bearing mice. PTHrP secretion into the medium of the tumour sublines grown in culture was measured in exponentially growing cells placed in serum-free medium for 20 h. As shown in Figure 3, PTHrP levels were highest in the 4T1.2 line, intermediate in 66cl4 cells and negligible in 67NR cells. Plasma PTHrP levels in tumour-bearing mice are shown in Table 2. Compared to the value in non-tumour-bearing mice, plasma PTHrP was elevated approximately 1.7 fold in mice bearing 4T1.2 tumours. This increase was significant ($p < 0.04$) when analysed using a Student t test.

Plasma calcium levels

Circulating calcium levels are presented in Table 2 along with the PTHrP data. In parallel with the elevation of plasma PTHrP levels in mice bearing 4T1.2 tumours, there was a significant increase in plasma calcium. In mice bearing 67NR tumours, calcium levels were also significantly elevated but to a lesser degree. No hypercalcaemia was evident in mice bearing 66cl4 tumours.

COLOR FIG.

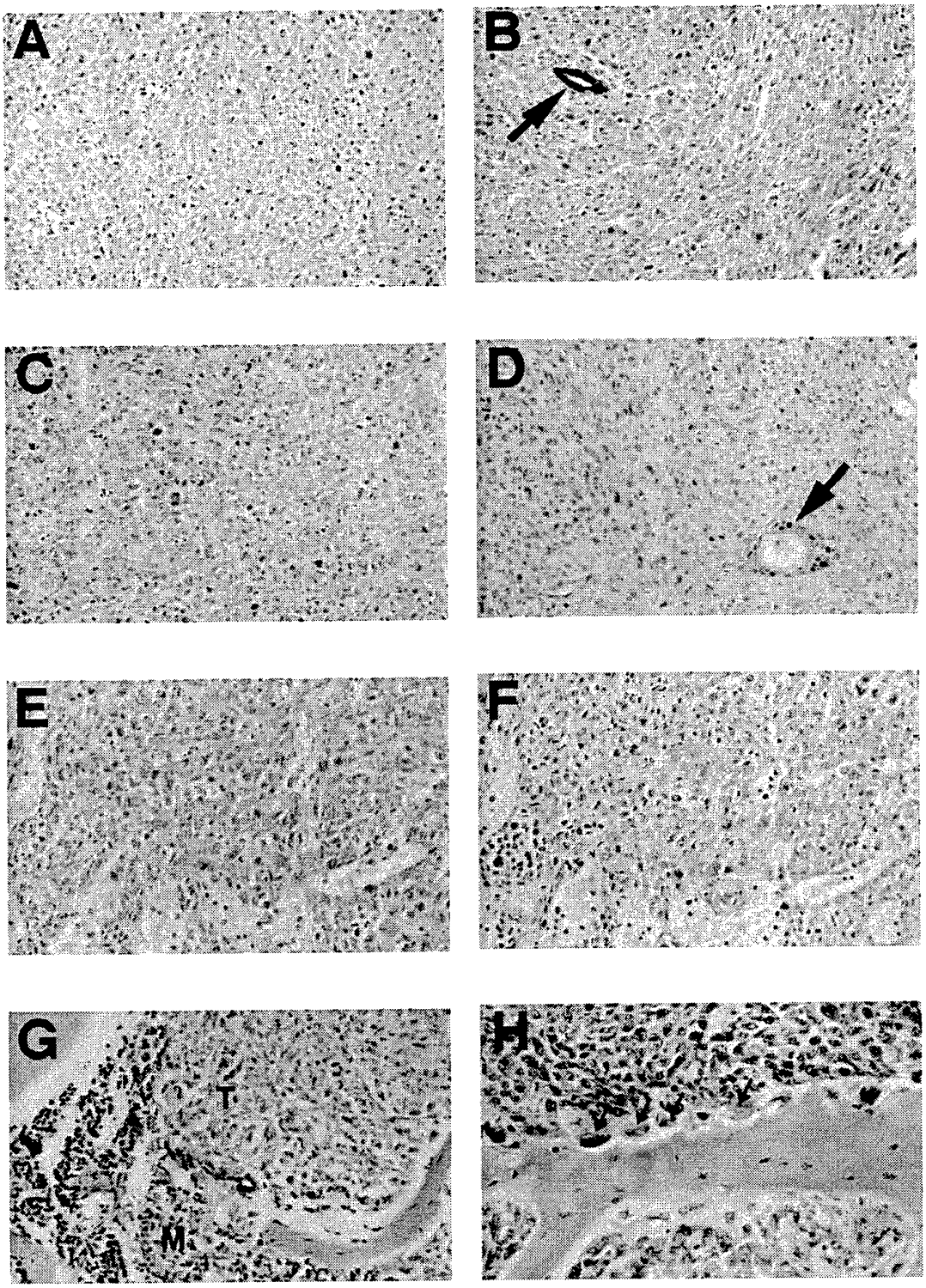

Figure 2. Morphological and immunohistochemical analysis of the 4T1.2 tumour. Section of the primary tumour showing morphological appearance by H&E staining (A) and immunostaining of the primary tumour for keratin (B), PTHrP (C) and estrogen receptor (D). Brown staining indicates regions of specific immunostaining. The arrows in **B** and **D** indicate strong immunostaining in epithelial cells lining the ducts. PTHrP immunostaining of a section through the femur, showing the growth plate and PTHrP positive tumour cells adjacent to it (E). Non-immune control of adjacent section of the femur to E (F). An H&E stained section through the spine showing a region of tumour (T) and a region of normal marrow (M) (G). **A–G** were photographed at ×80 magnification to show distribution of specific proteins throughout the tumour. A higher power image (×100) of a section of the spine stained with H&E showing the large multinucleated osteoclasts (indicated with arrows) adjacent to the trabecular bone, causing osteolysis of the bone (H).

Table 1. Tumour growth characteristics after injection of cells into the mammary fat pad or into the left ventricle.

Tumour subline	No. Mice	Time of growth (days, mean±SE)	Tumour weight (g) (mean±SE)	Lung metastases (mean±SE)	Bone metastases (No.+ve mice) Femur	Spine
Experiment 1						
67NR	7	32.6±0.4	2.3±0.8	0.2±0.4	0/3	0/3
66cl4	6	32.8±0.4	1.3±0.2	2.3±2.6	0/3	0/3
4T1.2	6	34.0±0	0.9±0.3	22.5±8.5	3/3	1/3
Experiment 2						
67NR	8	44.1±1.8	2.1±0.2	0	0/4	0/4
66cl4	14	30.6±1.4	1.2±0.1	15.7±1.6	0/4	0/4
4T1.2	15	36.6±0.9	1.0±0.1	34.3±2.7	4/4	3/4
Experiment 3						
67NR	5	17.0±0	–	12.2±6.0	0/4	1/4
66cl4	5	11.0±0	–	213.0±64.1	0/4	0/4
4T1.2	5	14.8±0.2	–	82.4±16.0	0/3	2/3

For the spontaneous metastasis assays shown in Experiments 1 and 2, 1×10^5 tumour cells were inoculated into the fourth mammary fat pad. Mice were culled when the primary tumour reached a size of approximately 1g or when the mouse first showed signs of distress. For the intra-cardiac experiment shown in Experiment 3, 2×10^5 tumour cells were inoculated into the left ventricle of the heart and mice were culled when they first showed signs of distress. Where appropriate, the primary tumour was excised and weighed. Lung metastases were scored after India ink inflation of the lungs. Tumour deposits in bone were detected by examination of H&E sections of the spine and femur.

Table 2. Plasma concentrations of PTHrP and calcium in mice bearing 67NR, 66cl4 and 4T1.2 tumours.

Cell Line	Plasma PTHrP (pM) Mean ± SE	Plasma Ca (mM) Mean ± SE
67NR	8.7±0.7 ($n = 15$)	2.33±0.03* ($n = 15$)
66cl4	4.7±0.5 ($n = 21$)	2.24±0.05 ($n = 20$)
4T1.2	12.1±1.5* ($n = 19$)	2.37±0.04** ($n = 13$)
Control mice	7.6±1 ($n = 13$)	2.21±0.02 ($n = 13$)

The samples were obtained from plasma taken when the mice were killed at the end of the experiment (Day 32–36 post inoculation). The '*n*' values indicate the number of mice analysed in each group. The *p* values were calculated using a two sample Student *t* test assuming unequal variances. Data with a single asterisk showed a p value < 0.05 whilst those with a double asterisk had a p value <0.005 compared to values obtained from control mice. The other data were not significantly different to values obtained in control mice. The controls were age matched, non-tumour bearing mice.

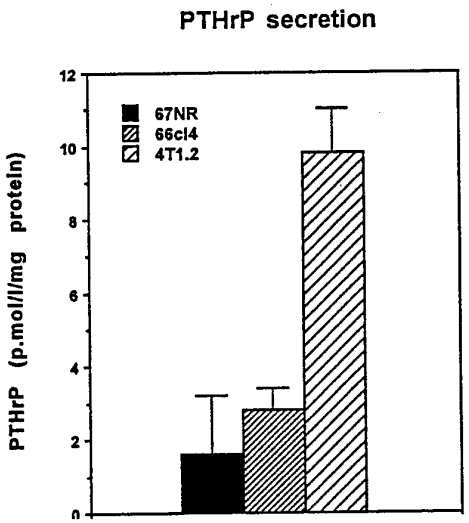

Figure 3. PTHrP secretion by the tumour sublines. PTHrP in the medium of cells in exponential phase of growth was measured by RIA and standardised to the protein content of the cells in the sample.

Discussion

Although much has been learned about the process of metastasis, less is understood of the mechanisms that dictate why tumours spread successfully to one particular site and not another. The establishment of a tumour in a tissue depends both on factors that it expresses and those released locally [16]. The concept that the microenvironment of an organ provides the fertile 'soil' for growth of a tumour cell was first proposed by Paget over one hundred years ago [17]. Bone is the favoured site of metastases of breast, prostate and lung cancers and thus the bone microenvironment must provide an appropriate fertile 'soil' for these tumours [16]. Once present in bone, tumour cells cause disruption of normal bone remodeling, with promotion of osteoclast formation and enhanced osteolysis usually occurring in the case of breast tumours. We have described here a clinically relevant model of breast cancer metastasis in which primary tumour cells spread from the mammary gland to bone and other distant sites. This model, with its use of subpopulations of

cells that metastasise from the primary site in the mammary gland to different organs, now provides the means to address the mechanisms of site-specific metastasis in a clinically relevant setting.

The 4T1.2 clonal cell line that we describe here metastasises to lung and bone following orthotopic inoculation into the mammary fat pad. The parental line 4T1 was shown previously to spread to lungs and liver [12] and here we show that clonogenic cells can be detected in spines of 4T1 bearing mice. Kinetic studies revealed that clonogenic cells could be detected in the lung prior to detection in the spine. As shown previously, the sister populations, 66cl4 and 67NR metastasised to the lung (66cl4) or did not metastasise at all (67NR). 4T1.2 cells generated tumours in both femur and spine and these were accompanied by local destruction of bone with active osteoclasts visible along bone surfaces. In none of our experiments with 4T1.2 did we observe tumours in the liver, although extramedullary hemopoiesis was observed.

Intracardiac inoculation confirmed that only the 4T1.2 cells were consistently metastatic to bone. This route of inoculation also led to an increased incidence of lung metastases in all three groups, including the normally non-metastatic 67NR tumours. No bone metastases were seen in mice inoculated with 66cl4 cells but spine involvement was observed in one of three mice inoculated with 67NR cells. This suggests that the 67NR cells may have some ability to grow in bone but are deficient in the mechanism required to detach from the primary site.

Our preliminary characterisation of this model has included use of keratin immunohistochemical staining to confirm the epithelial nature of the tumour cells and identification of ER in primary tumours generated from all three cell lines. Keratin staining was evident in all tumour sublines and was used as a guide to the identification of tumour cells at metastatic sites. The presence of the ER in the primary tumours, as evidenced by immunological staining, indicates an opportunity for the evaluation of anti-estrogen therapies in the modulation of site specific metastatic spread.

Since there is strong evidence implicating PTHrP in the metastatic spread of breast cancer to bone [18], we examined whether its expression correlated with bone metastasis in this model. PTHrP is the major mediator of hypercalcaemia in cancer patients including those with lung and breast cancers [19]. The primary tumours of all three cell lines investigated here stained positively for PTHrP, as did the bone metastases of 4T1.2 tumours. There were no major differences in the intensity of staining of this protein in the tumours generated from each of the cell lines indicating that, at the primary site, the presence of PTHrP in the tumour alone is insufficient to direct spread and growth specifically in bone. Nevertheless, levels of PTHrP secreted by the 4T1.2 cells *in vitro* were higher than those secreted by the 66cl4 or 67NR. Thus, it would seem that the higher level of cellular PTHrP secretion *in vitro* may be associated with the ability to generate bone metastases *in vivo*. Furthermore, levels of circulating PTHrP were raised significantly in mice bearing 4T1.2 tumours relative to controls and were higher than in 67NR and 66cl4 tumour-bearing animals. This may reflect increased release of PTHrP from tumour both at the primary site and in bone. Consistent with this and the known activity of PTHrP on bone was the observation that circulating calcium was slightly raised in animals inoculated with 4T1.2 cells.

The increased calcium levels observed in the 67NR tumour bearing mice, in which PTHrP levels were not raised, may reflect an alternative mechanism of osteolysis that could have facilitated tumour growth in the spine of one mouse inoculated via the intracardiac route with 67NR cells. Assessment of the expression and regulation of other bone resorbing cytokines in these cells will be of interest.

Whilst evidence for the role of PTHrP in the establishment of breast tumours in bone is convincing, it remains to be established whether other bone resorbing factors may be produced by the tumours to provide alternative or complementary mechanisms. Manipulation of the level of PTHrP expression by transfection or by clonal selection of the 4T1, 66cl4 and 67NR sublines will aid in addressing the importance of PTHrP secretion in directing site specific spread from the primary tumour, and its ability to be regulated in bone. We have derived clones of 4T1 with varying levels of PTHrP secretion and clones in which no PTHrP secretion can be detected. In experiments underway, the dependence on PTHrP expression for metastasis to bone and other sites is being tested using these clones. By analysing and comparing the gene expression profiles of the different sublines, we will be able to determine the contribution of other factors to the processes of homing and growth in bone. While this approach may endorse a role for PTHrP in promoting bone metastasis, it is likely to show that additional factors also have a critical function in homing to bone and in the development of bone metastases. In particular, it is likely that other bone resorbing cytokines, such as interleukin 6 [2] and interleukin 11 [4] may be as effective as PTHrP in enhancing bone metastasis formation.

This is the first natural model of mammary metastasis to bone to be reported. The model provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone, using genetically matched clones that do or do not metastasise to bone. An advantage of this unique system is that it uses subpopulations of a spontaneous metastatic tumour and represents the entire metastatic process from the primary site. The model is ideal for definition of the molecular mechanisms of site-specific metastasis and for the evaluation of therapeutic strategies such as anti-estrogens, and thus will contribute significantly to the clinical diagnosis and management of breast cancer patients.

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